Core Structures of Ubiquitin Dictate Its Dynamics and Function

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Mutations at solvent-inaccessible core positions may impact protein functions by changing protein dynamics. However, such mutations may also change the global structure and/or thermodynamic stability of proteins, and therefore, their impacts are difficult to be interpreted in many cases. In this issue of the *Journal of Molecular Biology*, Fushman, Bolon and their colleagues challenged this difficulty studying ubiquitin as the reference molecule.

Ubiquitin is a highly conserved 76-residue protein from yeast to mammals. The fold of ubiquitin consists of a five-stranded β-sheet, a short 3_{10} helix and an α helix [1]. The β-sheet is lined with the α helix to stabilize the overall fold. High solubility and thermostability [2,3] make ubiquitin a standard molecule for investigating protein structure and dynamics by physicochemical analyses (summarized in the introduction of Ref. [4]). Biologically, ubiquitin plays critical roles in control of various cellular processes through covalent attachment to substrate proteins [5,6]. In many contexts, one ubiquitin molecule is linked to the other ubiquitin molecule to produce polyubiquitin chains to exert their functions. A prominent example is protein degradation by the proteasome, where polyubiquitin chains linked via Lys48 serve as the degradation signal [5]. The enzymatic cascade of (poly)ubiquitination includes a series of enzymes: Ubiquitin is activated by a ubiquitin-activating enzyme E1. Then, a ubiquitin-conjugating enzyme E2 receives the activated ubiquitin. Finally, a ubiquitin ligase E3 aids transfer of the ubiquitin to substrate proteins. Many ubiquitin receptors contribute to recruitment of substrate proteins to the proteasome. Polyubiquitin chains are detached from substrate proteins by proteasome-associated deubiquitinating enzymes before proteolysis occurs and then efficiently recycled as free ubiquitin molecules. Therefore, a balance between free and conjugated states of ubiquitin should be tightly coupled with its function in cells. If certain mutations at core positions could change this balance without any impact on the overall structure and thermostability, such mutations might affect ubiquitin function by altering the dynamics.

Fushman, Bolon and their colleagues investigated relationship of ubiquitin core structures with dynamics and function in ubiquitin by using spectroscopic techniques of nuclear magnetic resonance (NMR) and circular dichroism (CD) and *in vivo* growth assay that they developed to study impacts of ubiquitin mutations on yeast cells [7]. Ubiquitin expression in the previously developed SUB328 yeast strain strictly depends on galactose. Therefore, replacement with dextrose media rapidly decreases ubiquitin expression level and stops the yeast growth, which can be recovered by introducing a plasmid that constitutively express ubiquitin. All 15 core residues of ubiquitin were assessed by this rescue experiment. Of 15 ubiquitin mutants, 13 could fully or partly support yeast growth, whereas I30A and L43A mutants failed. Thermodynamics of these mutants and other growth-supporting mutants was investigated by CD spectrometry. Their melting temperatures estimated from the CD spectra indicate that I30A and L43A mutants are well folded in yeast cells, similarly to wild type and other growth-supporting mutants.

Behaviors of the ubiquitin mutants were also surveyed by checking accumulation of free ubiquitin monomers and covalently conjugated high-molecular-weight ubiquitin species in yeast cells. Most of the mutants including I30A are accumulated as both free ubiquitin and high-molecular-weight species. By contrast, L43A mutant is accumulated as only a reduced amount of high-molecular-weight species in cells, which disappear when the conjugation is disabled, suggesting that L43A mutant may be degraded together with substrate proteins by the proteasome

without being detached from the substrate proteins (Fig. 1). Therefore, L43A mutation affects ubiquitin function independently of its thermostability but potentially changing its dynamics.

The overall structure and backbone dynamics of L43A mutant were investigated by NMR spectroscopy. The $^{1}H–^{15}N$ heteronuclear single quantum coherence spectra of L43A mutant and wild type suggested a little difference in a hydrophobic core between L43A and wild-type ubiquitin. However, residual dipolar couplings for L43A mutant that were observed in a weakly aligned liquid crystalline medium exhibit excellent agreement with those calculated from the wild-type ubiquitin structure, indicating that their overall structures are very similar to each other. On the other hand, ^{15}N relaxation experiment demonstrated that slower, millisecond-to-microsecond motions are affected by L43A mutation, although faster, sub-nanosecond motions are similar between wild type and L43A mutant. Further NMR analyses in the presence of ubiquitin receptors showed that L43A ubiquitin mutant is recognized by proteasome-associated ubiquitin receptors in the same stoichiometry and manner as wild type. Similarly, *in vitro* assays of assembly and disassembly of polyubiquitin chains showed that L43A mutants are compatible with the assembly and disassembly, as well as binding to ubiquitin receptors.

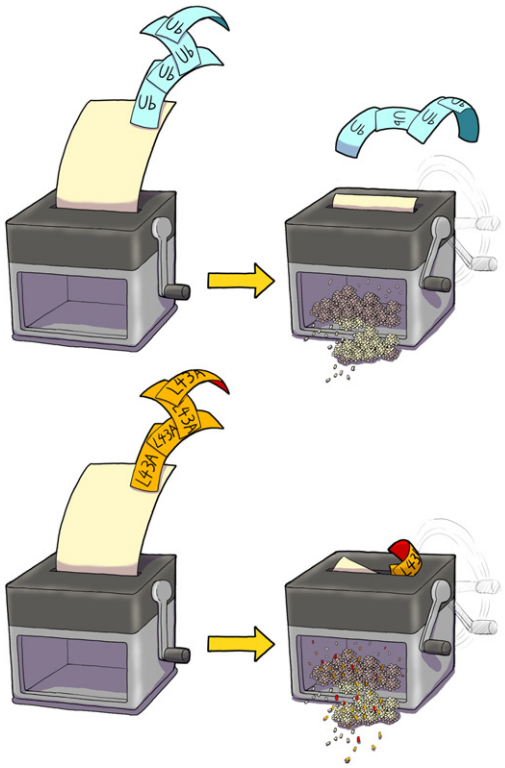

Fig. 1. Threading of ubiquitin-conjugated substrates into the internal cavity of the proteasome.Wild-type ubiquitin (drawn as cyan sticky notes) is detached from substrate proteins (drawn as a sheet of paper) before threading and degradation by the proteasome (drawn as a shredder), whereas L43A mutant (drawn as orange sticky notes) is not detached from substrate proteins and threaded into the cavity together with substrate proteins.

In conclusion, L43A mutation alters the dynamics of ubiquitin with little change in the global structure, thermostability and abilities for ubiquitin-receptor binding and assembly/disassembly of polyubiquitin chains. The altered dynamics causes unusual accumulation pattern of L43A mutant in yeast, suggesting a rapid turnover of L43A ubiquitin mutant. Given a mechanistic model for recognition and processing of ubiquitin-protein conjugates by the proteasome [8–10], the rapid turnover of L43A mutant is possibly due to more rapid threading of L43A mutant into the internal proteasome cavity and degradation than wild type (Fig. 1). The authors further speculate that threading of substrate proteins into the cavity is obstructed when the substrate-conjugated (proximal) ubiquitin is encountered and that wild-type ubiquitin causes stronger obstruction than L43A mutant because of the higher stability. Direct measurements of the recycling efficiency and half-life of ubiquitin mutants including L43A may help to support this speculative hypothesis.

This study demonstrated that a combination of *in vivo* alanine-scan screening of ubiquitin and *in vitro* biophysical spectroscopic analyses could identify an amino acid residue that alters ubiquitin dynamics with little change in the global structure and thermostability. This combinatorial approach should not be limited to ubiquitin but generally applicable to other proteins for finding core residues that dictate protein dynamics and function. Information of such core residues and their environments might provide clues as to how core structures elaborately control protein dynamics and function in general and, further, why disease-related mutations of proteins are found not only in functional sites on the surface but also in core regions.

Commentary to the featured article by Lee et al., “Alanine scan of core positions in ubiquitin reveals links between dynamics, stability, and function”.

References

[1] Vijay-Kumar S, Bugg CE, Cook WJ. Structure of ubiquitin refined at 1.8 Å resolution. J Mol Biol 1987;194:531–44.
[2] Herberhold H, Winter R. Temperature- and pressure-induced unfolding and refolding of ubiquitin: a static and kinetic Fourier transform infrared spectroscopy study. Biochemistry 2002;41:2396–401.

[3] Wintrode PL, Makhatadze GI, Privalov PL. Thermodynamics of ubiquitin unfolding. Proteins 1994;18:246–53.
[4] Piana S, Lindorff-Larsen K, Shaw DE. Atomic-level description of ubiquitin folding. Proc Natl Acad Sci U S A 2013;110:5915–20.
[5] Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82:373–428.
[6] Grabbe C, Husnjak K, Dikic I. The spatial and temporal organization of ubiquitin networks. Nat Rev Mol Cell Biol 2011;12:295–307.
[7] Roscoe BP, Thayer KM, Zeldovich KB, Fushman D, Bolon DN. Analyses of the effects of all ubiquitin point mutants on yeast growth rate. J Mol Biol 2013;425:1363–77.
[8] Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 2009;78:477–513.
[9] Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003;426:895–9.
[10] Prakash S, Inobe T, Hatch AJ, Matouschek A. Substrate selection by the proteasome during degradation of protein complexes. Nat Chem Biol 2009;5:29–36.